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RESEARCH

The Determination of Cisplatin and Luteolin Synergistic Effect on Colorectal Cancer Cell Apoptosis and Mitochondrial Dysfunction by Fluorescence Labelling

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Abstract
Despite the initial success of chemotherapy in the treatment of colorectal carcinoma (CRC), the recurrence of the disease shows that the tumor response is limited by the formation of drug resistance and cannot be kept under control. These drawbacks are associated with the cytostatic chemotherapeutic agent cisplatin (Cis). Combination treatment with different anticancer drugs could increase the therapeutic efficacy of combined therapies by allowing the use of lower, less toxic doses to achieve more efficient destruction of cancer cells. Luteolin (LU) has been studied with other anticancer drugs due to its anticancer cell inhibitory properties and has been shown to sensitize the cytotoxicity induced by various anticancer drugs in several cancer cells. Therefore, in this work, the CompuSyn system was used to investigate different Cis + LU combinations in HCT116 colorectal cancer cells. Immunofluorescence was used to measure mitochondrial membrane potential (MMP) and cell death. As a result, a synergistic effect was observed in 5 of the 7 doses tested. Apoptosis/necrosis resulting from chemotherapy resistance was confirmed by Hoechst/ PI -double staining and mitochondrial dysfunction were determined by Rodamine123 (Rho123). Luteolin could thus be used in medicine to provide more effective cancer therapy in appropriate doses, which promises a promising future in clinical application.

Keywords Cisplatin · Luteolin · Synergism · Colorectal cancer

Introduction

Many therapeutic plant species, including carrots, peppers, celery, olive oil, mint, thyme, and rosemary, contain luteolin, a flavonoid group known as 3',4',5,7-tetrahydroxyflavone [1, 2]. Due to its anti-inflammatory, antiallergenic, and anticancer characteristics, luteolin is utilized in the treatment of numerous disorders including hypertension, inflammatory diseases, and cancer. It is both pro- and antioxidant in nature. Additionally, it was discovered at the end of the experiments that luteolin causes cancer cells to undergo apoptosis, inhibits cell proliferation, and prevents metastasis and angiogenesis [3–5]. Much recent research indicates that luteolin has the potential to be administered in cancer treatment [6, 7]. Furthermore, it has been suggested that a luteolin-rich diet can be used to prevent several chronic diseases as well as cancer [7].

Cisplatin is a platinum-based anticancer medication that is frequently used in the treatment of several cancers, including colorectal, testicular, ovarian, head and neck, bladder, and lung [8]. Besides many mechanisms, cisplatin causes anticancer activity mainly because of its interaction with DNA [9]. The interaction of cisplatin with DNA is thought to interrupt the cell cycle and trigger apoptosis [10]. Patients who respond successfully to cisplatin at the start of cancer treatment, on the other side, generally acquire resistance to the drug in the later phases of treatment. Although cisplatin is an effective medicine, dose-related adverse effects, particularly nephrotoxicity, and cancer cell resistance to cisplatin have a negative impact on cisplatin treatment [11]. Furthermore, dose and time dependent hepatotoxicity and testicular toxicity are documented side effects [12]. The development of chemosensitization approaches has become an important goal, particularly because cisplatin is the primary therapeutic choice in various cancer treatments [9].

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Many investigations are being conducted for the anticancer medication cisplatin to provide more effective therapy. New therapeutic approaches are being sought to diminish cisplatin resistance and toxicity. Recently, innovative treatment techniques that integrate the use of two or more medicinal substances have piqued the interest of researchers [13–15]. The search for novel therapeutic alternatives continues to diminish cisplatin resistance while minimizing toxicity. Recently, innovative treatment techniques that integrate the use of two or more therapeutic agents have gained attention. Combination treatment is the use of one anticancer drug in conjunction with another agent, and it has the potential to diminish drug resistance in general. Supplementing anticancer treatment with natural chemicals may give a more effective treatment in reducing cancer cell growth and spread by triggering apoptosis and may even diminish the cancer stem cell population [16, 17]. The survival rate is extremely poor, especially in metastatic cancers such as colorectal cancer [18]. New effective therapeutic options to improve survival in certain cancer types are being researched, but the product development process is both expensive and time-consuming. More effective treatment, particularly for anticancer drugs with severe side effects and drug resistance development, can be generated using substances with natural anticancer capabilities such as luteolin. For these reasons, research into natural chemicals known to have anticancer properties as combination therapeutic agents is critical in establishing novel and successful treatment regimens. Drugs that target the key pathways in a synergistic manner boost the efficacy of combination therapy and represent a viable treatment alternative. In this paper, colon cancer cells (HCT116), an extremely aggressive kind of malignancy, were employed. Cisplatin is an anticancer medicine that is commonly used to treat colon cancer and has a significant risk of developing drug resistance. For these reasons, we explored adverse effects of cisplatin and drug resistance therapy options. We evaluated the apoptotic effects of cisplatin and luteolin in combination with Hoechst33342 and PI dyes, as well as drug resistance with Rho123 activation.

Materials and Methods

In Vitro Experimental Design

The HCT116 human colon cancer cell line and BEAS-2B normal human bronchial epithelium were cultured in DMEM media containing 5% fetal bovine serum and 1% Penicillin-G (100 U/mL)-Streptomycin (100 µg/mL) until the required number of cells were attained. 5×10^3 cells were sown into 96 wells and incubated in 5% CO_2 at 37 °C. Cisplatin and luteolin were applied in six different concentrations. TDC values for cisplatin were obtained using pharmacokinetic/clinical data and tentative clinical interpretation of testing findings [19]. TDCs match to the plasma concentrations obtained in vivo after a standardized dosage of every drug analyzed [20] and enable for the discovery of dose–response effects [21]. The concentrations employed were 200% TDC, 100% TDC, 50% TDC, 25% TDC, 12.5% TDC, and 6.12% TDC. Cisplatin 100% TDC value is µg/mL. HCT116 cells were treated six different luteolin doses: 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 The HCT116 human colon cancer cell line was cultured in DMEM media containing 5% fetal bovine serum and 1% Penicillin-G (100 U/mL)-Streptomycin (100 g/mL) until the required number of cells were attained. 5×10^3 cells were sown into 96 wells and incubated in 5% CO_2 at 37 °C., 6.25 µg/mL, and 3.125 µg/mL. Sulforhodamine B (SRB) analysis was used to determine the individual effect of cisplatin and luteolin on colon cancer cell line (HCT116) viability after 48 h of incubation. SRB detects living cells primarily by stoichiometrically binding to proteins [22]. The IC_{50} (concentration that inhibits 50% of cell growth) values of compounds were calculated using dose/response curves. The extracts' efficacy was measured against this criterion.

The optimal anti-proliferative dosage of cisplatin was reported to be 200% TDC value (7.6 g/mL). The SRB test was used to assess the cytotoxic impact of 7.6 g/mL cisplatin and luteolin, created by serial dilution of all dosages, on the human colorectal cancer HCT116 cell line. The SRB test was performed after 48 h of incubation of HCT116 cells grown at 5000 cells/well. Because of the SRB test, the cytotoxic impact was measured using spectrophotometric measurements. To investigate the effects of cisplatin, luteolin, and their combination on the HCT116 human colorectal cancer cell line and BEAS-2B normal human bronchial epithelium, 96-well cell culture plates were seeded with three separate wells for each dosage. Each experiment was conducted as at least two different independent experiments. The GraphPad Prism5 program was used to calculate the spectrophotometric measurement findings acquired because of the SRB technique used for cytotoxicity determination.

Combination Analyzes of Cisplatin and Luteolin

Furthermore, different concentrations of cisplatin and luteolin were mixed in HCT116 colon cancer cells, and the combined effects were assessed using the Compusyn method. In drug combination studies, according to the combination index (CI) theorem, $CI = 1$ indicates additive effect, $CI < 1$ indicates synergism, and $CI > 1$ indicates antagonism [23].

HOESCHT33342/Propidium Iodide (PI) Double Staining

Given the shape of the cell, DNA is uniformly distributed throughout, and the nuclei are typically spherical. However,

DNA condenses and a pycnotic look appears during apoptosis. Nuclear differences can be utilized to separate apoptotic cells from healthy cells and necrotic cells since this does not happen during necrosis. Investigations into nuclear condensation can be conducted using DNA-binding dyes like Hoechst 33,342 [24]. Hoechst 33,342 and propidium iodide staining are often used together for simultaneous flow cytometric and fluorescence imaging analysis of the stages of apoptosis and cell-cycle distribution. HCT116 cells were seeded at 5000 cells/100 µl per well in 96-well plates. Non-treated HCT116 control group, luteolin or cisplatin treated HCT116, and all combinations of luteolin and cisplatin administered HCT116 groups were given IC_{90} (dose reducing cell viability by 90%) values, and after 24 h, Hoescht33342 (ThermoFisher) and Propidium Iodide (PI) (BioLegend) staining protocol was used. 50 µL of each dye were added to the wells, and supernatants were taken from the working solution prepared at 1 g/mL and incubated in the dark at room temperature. The images were captured using a Nikon Eclipse Ts2 immunofluorescence microscope.

Rhodamine 123 Staining

Rhodamine 123 (Rho123) staining was used to perform the mitochondrial membrane potential assay in non-treated HCT116 control group, luteolin or cisplatin treated HCT116, and cisplatin-luteolin combinations treated HCT116 groups. All groups were fixed with 3% glutaraldehyde solution in 1 × PBS 24 h after being treated with all cisplatin-luteolin combinations. After preparing a 10ug/mL working solution of Rhodamine123 in 1X PBS, all groups were stained and incubated in the dark for 30 min at 37 °C. Nikon Eclipse Ts2 immunofluorescence microscope was used to take photographs after dehydration and mounting.

Ethical Statement

HCT116 and BEAS-2B cell lines were obtained from Istanbul University Faculty of Medicine, Physiology Department. Since they are commercially produced cell lines, no ethics committee approval is required.

Statistical Analysis

Cell viability graphs and IC_{50} values were determined using the GaphPad Prism 5 Programme.

Results

The cytotoxic effects of cisplatin, an anticancer drug, and luteolin, a flavonoid molecule, were assessed in individual HCT116 colon cancer cells using the SRB test shown in Fig. 1. The IC_{50} value for luteolin was 15.05 and 5.8 µg/ml for cisplatin shown in Table 1.

In this work, a total of 7 data/dose point was investigated. The large-scale constitution methodologies clearly demonstrate that automatically generated computer simulations from the dose and impact collected data allow pharmacological graphic modifications to the dose–effect curves

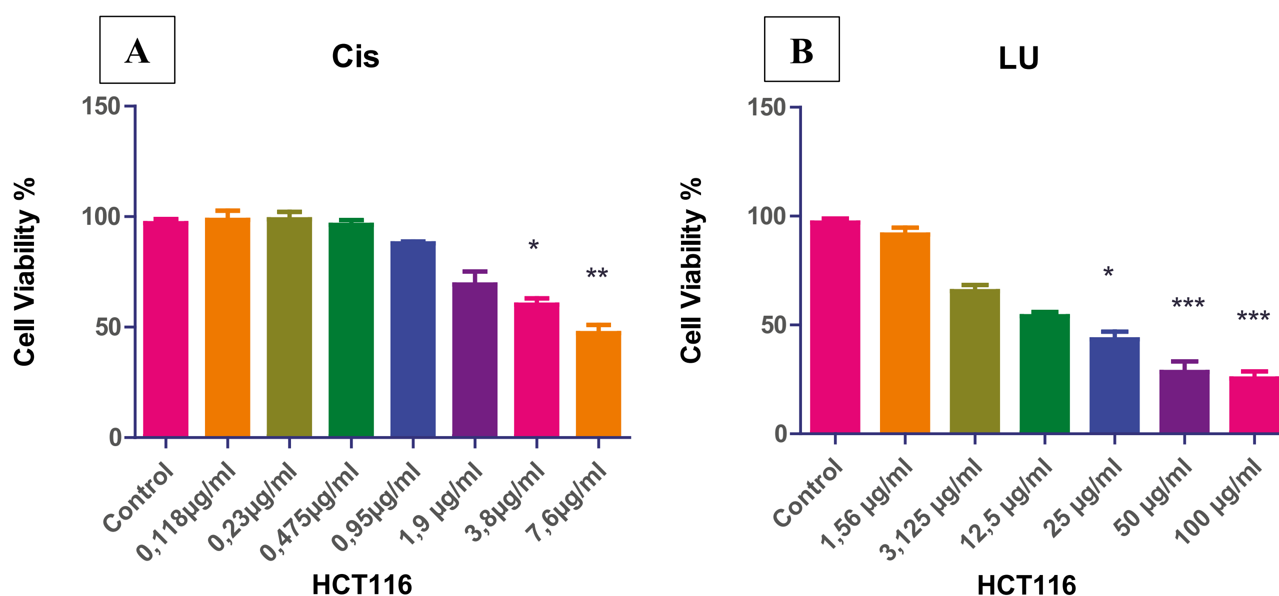

Fig. 1 Cell viability % of **A** cisplatin, **B** luteolin. It was evaluated by Graphpad Prism software. The viability rates of cisplatin or luteolin at the end of 48 h were compared with the control group (***$p < 0.001$; ** $p < 0.01$; *$p < 0.05$)

Table 1 IC_{50} values of LU and Cis on HCT116 on colon cancer cells

Compounds	IC_{50} values (μg/mL)
LUT	15.05
Cis	5.8

Table 2 Indication of Lu/Cis Combination Index (CI) and results are shown

Groups	Dose Lu	Dose Cis	Effect	CI	
0	50.0	2.0	0.28	1.16473	Antagonism
1	25.0	5.0	0.29	0.90514	Synergism
2	25.0	1.0	0.31	0.69354	Synergism
3	12.5	2.0	0.44	0.88623	Synergism
4	12.5	1.0	0.5	1.00136	Additive
5	6.25	5.0	0.34	0.63173	Synergism
6	6.25	2.0	0.47	0.66217	Synergism

(Fig. 2A), to the linearized median-effect plots (Fig. 2B). After analyzing the combination index and logarithmic combination index, it is evident that only one value is in the antagonistic zone, while the other is in the additive region. The other 5 combinations, on the other hand, are in the synergistic area (Fig. 2C, D). At varying doses (except 4), the combination of cisplatin and luteolin demonstrated a synergistic effect with a typical CI value of 1 (Table 2).

We aim to assess the impact of these combination dosages on BEAS-2B healthy cells after determining these doses. These cells are depicted in Fig. 3 with no notable change. The combination research on colorectal cells was successful in producing these outcomes in healthy cells, demonstrating its applicability.

As shown in Fig. 4, the administration of luteolin, cisplatin, and combination Cis + LU results in nuclear alterations in HCT116 cells after Hoechst33342 and PI staining. Hoechst33342 binds to nuclear DNA of cells. Based on IC_{90} levels, each combination of Cis + LU resulted cell death significantly. Apoptotic cells are more prevalent in combinations 2 and 4, whereas late apoptosis/necrotic cell groups

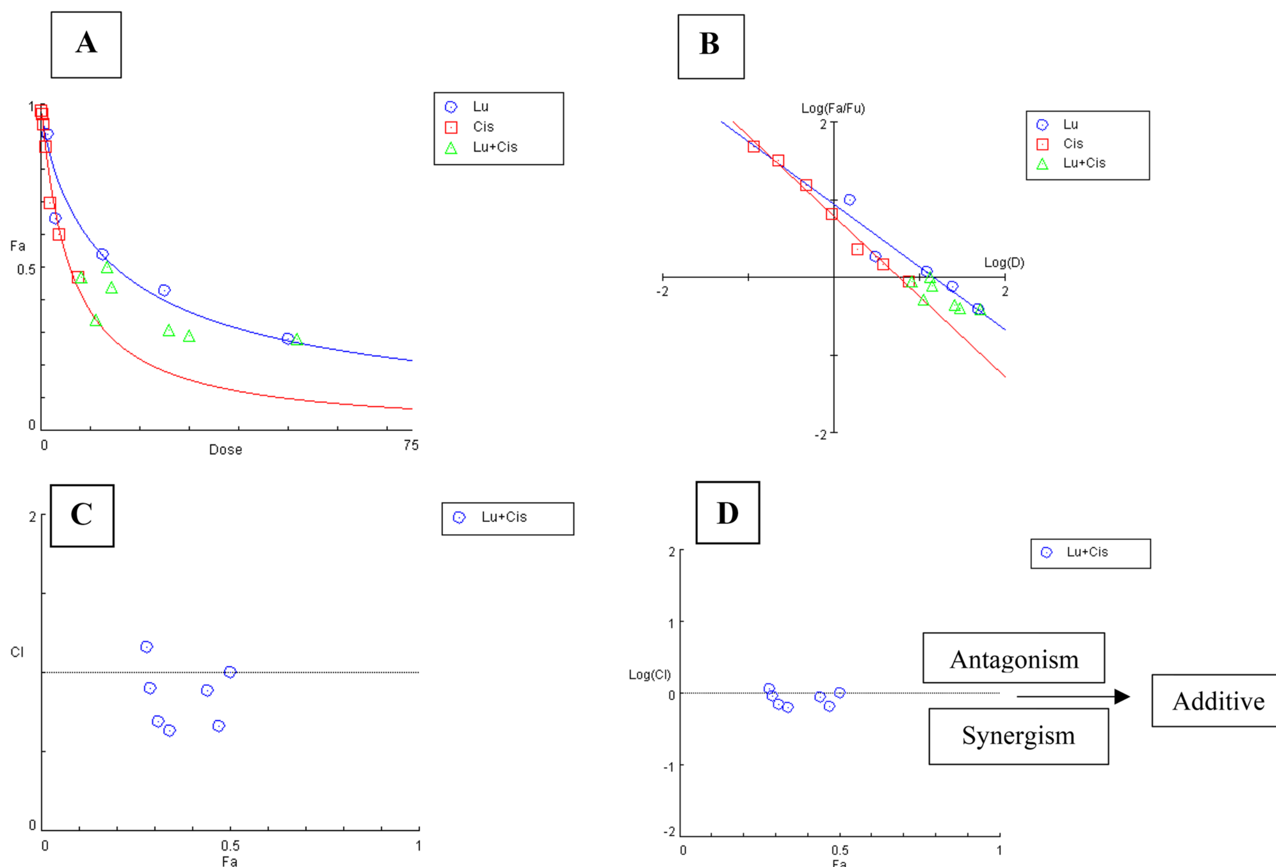

Fig. 2 Using cisplatin, luteolin, and cisplatin-luteolin implemented groups, the mass-action law algorithms based pharmaco-dynamic computerized transformations and depiction in four diagnostic graphic forms were created. **A** Dose–effect curve. **B** Median-Effect Plot. **C** Combination-Index Plot. **D** Logarithmic Combination-Index Plot

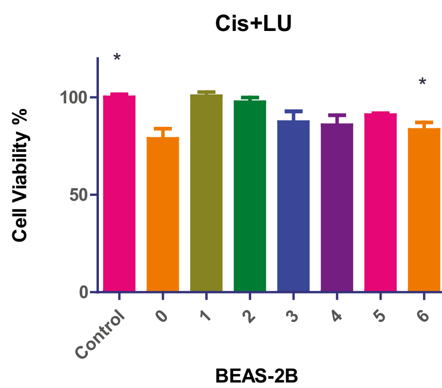

Fig. 3 The cell viability % of Cis + LU combination doses (0–6) treated BEAS-2B cells are seen. The viability rates of cisplatin and luteolin combinations at the end of 48 h were compared with the control group (***$p < 0.001$; ** $p < 0.01$; *$p < 0.05$)

predominate in all other groupings. Pycnotic cell nuclei was the characteristic of these groups (Fig. 4).

The construction of a mitochondrial permeability transition hole results in membrane potential loss, phosphorylation dysfunction, ATP deprivation. It is known that apoptosis is a process that causes the cell and its organelles to condense and shrink, eventually resulting in cell fragment. The shrinking of the mitochondria is one of the hallmarks of a late apoptosis. Mitochondrial membrane potential could be measured via rho123 staining. In cells with a high membrane potential, green fluorescence is detected. We discovered that treatment with cisplatin-luteolin combinations reduced the mitochondrial membrane potential in HCT116 cells, as demonstrated by a reduction in fluorescence relative to control groups (Fig. 5).

Discussion

Cisplatin exerts anticancer activity primarily through its interaction with DNA, among other mechanisms [25]. Patients who respond successfully to cisplatin at the start of cancer treatment, on the other hand, acquire resistance to the drug in the later phases of treatment. Although cisplatin is an effective medication, dose-related toxicity, and resistance of cancer cells to cisplatin have a detrimental impact on cisplatin treatment [26]. Many experiments are being conducted in attempt to improve the efficacy of the anticancer medication cisplatin [27–29]. The search for innovative therapy alternatives to diminish cisplatin resistance and toxicity continues [14]. In cancer treatments, medication combination therapy can increase therapeutic efficacy, minimize drug dose, and reduce drug resistance. Many theoretical and empirical methods for identifying pharmacological combinations that work synergistically rely on clinical or practical expertise and scanning preset drug libraries. Drugs that target the key routes synergistically improve the efficacy of combination therapy and offer a viable therapeutic alternative [30]. With a limited dataset, the CI approach systematically calculates synergism or antagonism at certain dosages and those efficacy factors [30–32]. In numerous cancer trials, cisplatin has been combined with excessive amounts of flavonoids to counteract the negative effects of the disease, such as drug resistance, colitis, mucositis, and pain in colorectal cancer. Cisplatin interaction with quercetin has undergone significant research [33–36]. As a result, flavonoids can lessen the impact of chemotherapy on CRC. Even Wang and his colleagues demonstrated how luteolin induces apoptosis and inhibits cell migration and invasion to increase the anticancer impact of cisplatin in drug-resistant ovarian cancer [37]. The fact that 5 out of the 7 combinations we tested in our study had a synergistic impact is highly noteworthy. We believe that the antagonistic dosage of 0 was caused using a cisplatin dose greater than its IC_{50} value. The lack of harm to healthy cells and the selective cytotoxicity of the drugs are key factors in the efficacy of cancer treatment. On healthy cell lines, BEAS-2B, Cis + LU combination dosages of 0–6 were attempted in an effort to comprehend this, but no appreciable effect was seen on the percentage of cell viability. This outcome demonstrates the likelihood that the dosages we employ may really be employed for the best cancer treatment.

One of the primary mechanisms behind chemotherapy resistance is apoptosis suppression [38]. After the apoptotic pathway is disrupted, tumor cells develop resistance to pro-apoptotic impact of Cis, which lessens cytotoxic effects of it [39]. As a result, suppressing apoptosis is a successful tactic for combating drug resistance and enhancing cisplatin's anti-carcinogenic effects [40]. Human cervical cancer cells [41], esophageal carcinoma cells [42], and colorectal cancer cells [43] are just a few of the cancer cells that luteolin has been shown to trigger apoptosis in. All dead cells, whether they are primary necrotic or late apoptotic/secondary necrotic, are stained by PI, a fluorescent nucleic acid dye that can only enter membrane-damaged cells. Living cells discharge this dye. The traditional kind of death that takes place under toxic settings is primary necrosis (increased cell volume but no fragmented or pycnotic nuclei) (hypoxia, ischemia, hyperthermia, etc.). The later stage of apoptosis, secondary necrosis is marked by pycnotic or shredded nuclei. The membrane integrity of the cells is compromised with the development of late apoptosis/secondary necrosis in later periods, even though the membranes of cells undergoing apoptosis in cell culture media are intact (in early apoptosis). Even though apoptosis has begun, the cells cannot be stained with non-vital dyes (PI) since the membrane is still intact throughout the time leading up to the secondary necrosis stage. The cells start to stain with non-vital dyes when the integrity of their membranes deteriorates in the phases following the development of secondary necrosis. As a result, they exhibit PI

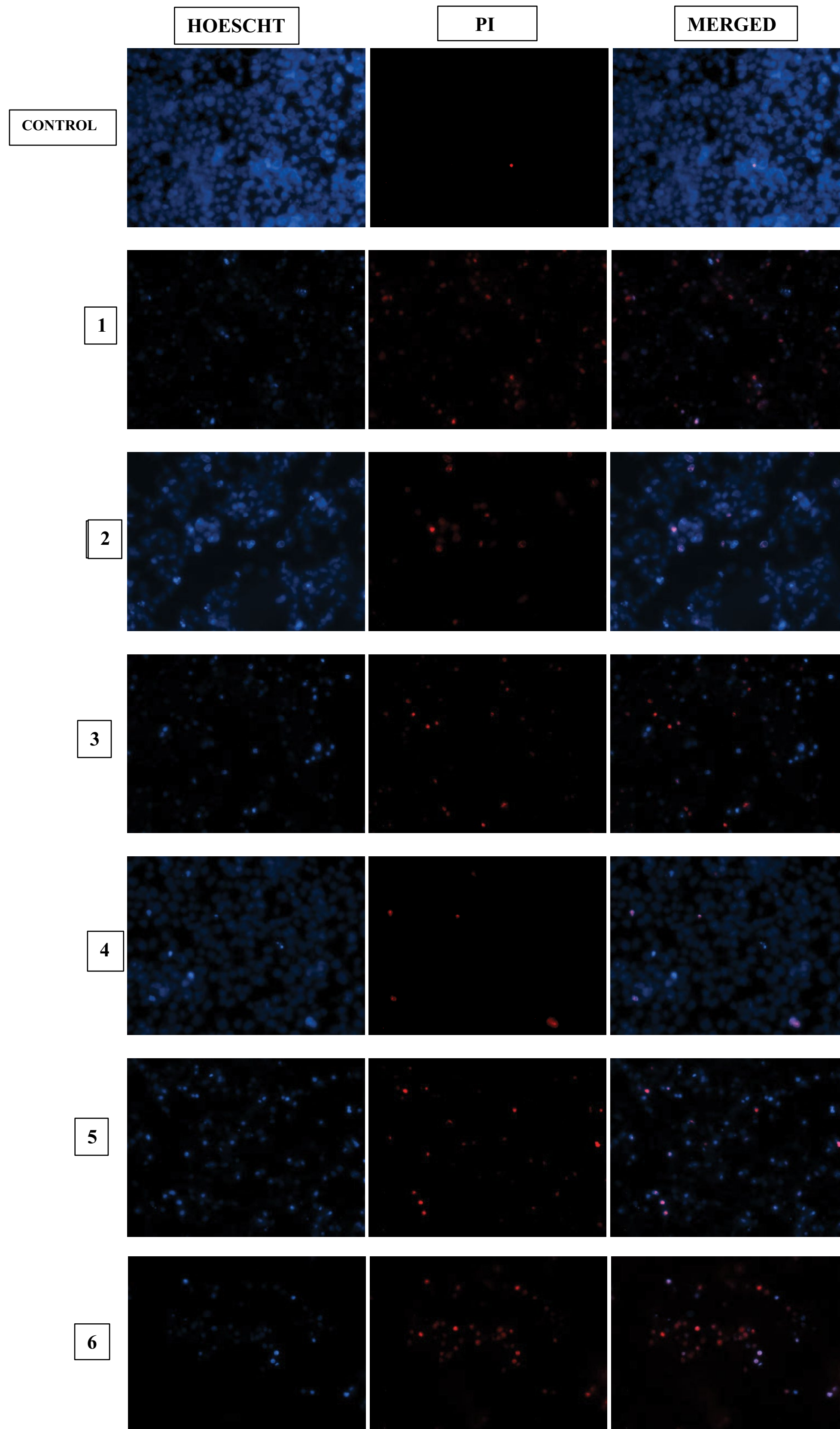

Fig. 4 Hoechst 33,342 and propidium iodide (PI) staining of luteolin, cisplatin in IC_{90} values, and luteolin + cisplatin in different concentrations on HCT116 cells. Scale Bars:10X

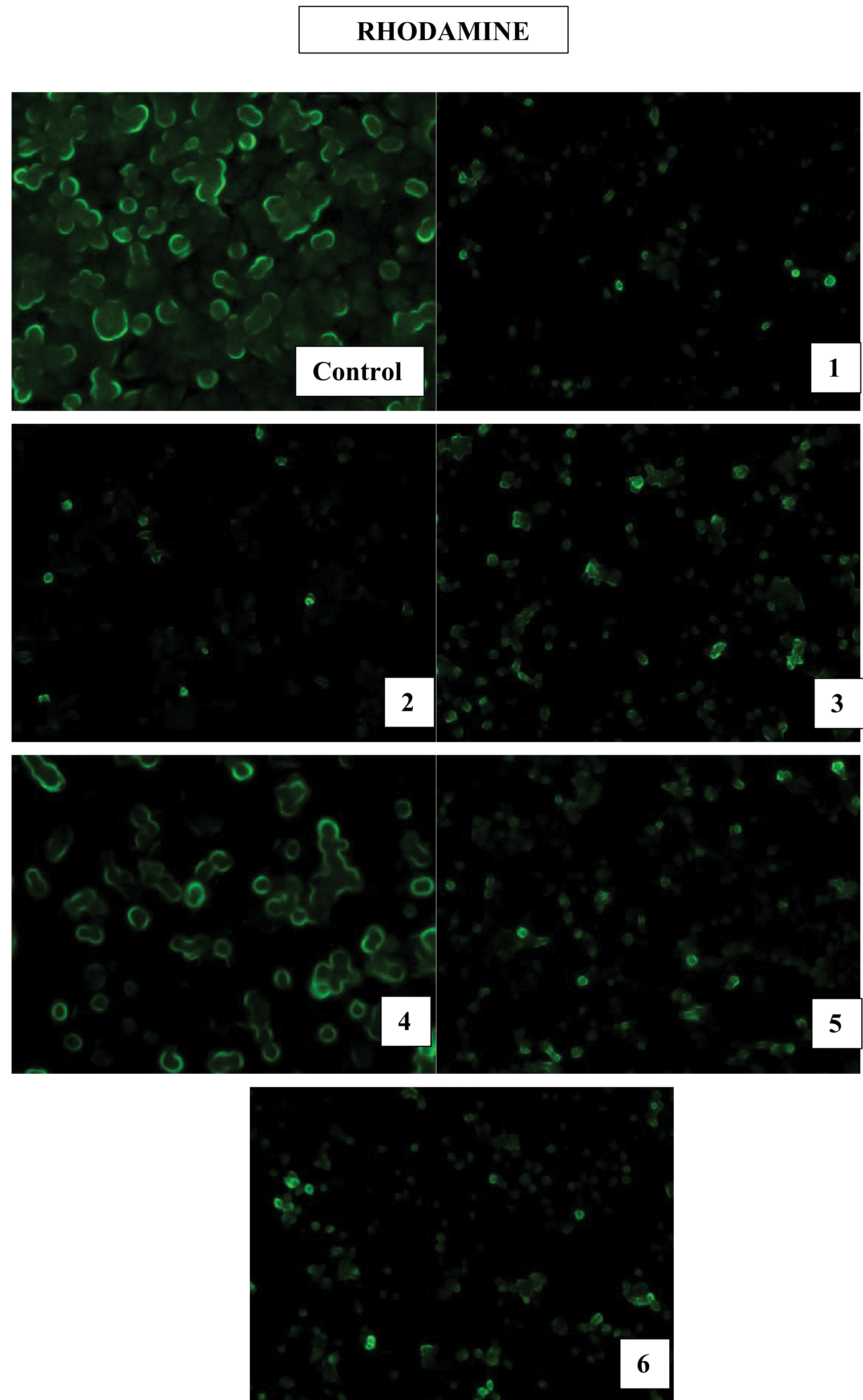

Fig. 5 Mitochondrial shrinkage which is related with late apoptosis and lesser fluorescence labelled mitochondria were seen in all combinations of the Cis + LU-treated HCT116 groups, except for groups 2 and 4. Scale Bars: 20X

and Hoechst positivity [44]. Our findings indicate that, apart from the 2nd and 4th combination groups, there was substantial necrosis present in the IC_{90} values of the dosages we utilized. This demonstrates that using cisplatin and luteolin combined at lower dosages might make sense based on TDC values of cisplatin.

Mitochondrial changes are crucial signals that reveal the stress and health of a cell. One of the regulators that plays a significant part in the apoptosis process is the mitochondria. The irreversible stage in the apoptotic process is indicated by mitochondrial activation (the release of cytochrome c from mitochondria to cytoplasm), which is a junction point at the intersection of apoptotic pathways [45]. Therefore, after inducing apoptosis, cells show a decrease of mitochondrial integrity. When taken to its furthest, such shrinking results in the full loss of the mitochondrial genome, which has a negative impact on the organelle's structural complexity and metabolic flexibility [46]. In our analysis, we found that all cisplatin-luteolin combos were particularly associated with late apoptosis showed mitochondrial shrinkage. This suggests that in these populations, the potential of the mitochondrial membrane has reduced. Additionally, cancer medications may be carried out of cancer cells because of multidrug resistance-related protein (MRP) expression, which lowers effective doses [47]. It was reported that luteolin overstimulates two Oxaliplatin-resistant colorectal cancer cell lines to chemotherapeutic treatments through disruption of the Nrf2 pathway [48].

Conclusion

New alternative therapies for patients have emerged because of the frequent negative effects of chemotherapy medications. Combination treatment has gained popularity recently and has positive outcomes. By combining various dosages of cisplatin, our study compared the effects of luteolin, a substance with extremely significant qualities, against apoptosis, necrosis, and mitochondrial membrane potential. Therefore, it is crucial that HCT116 cell groups exhibit synergism for 5 of the 7 dosages that were employed. Additionally, it shows how effective the combinational techniques may be when the morphological degenerations in the cells that we utilize are comparable in the same groups that have a synergistic impact with the binary combinations we use.

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Author Contributions Concept, design, supervision, sources, data collection and processing, analysis and interpretation, literature review and writing the manuscript is done by D.Ö.

Availability of Data and Materials Not applicable.

Declarations

Ethical Approval HCT116 and BEAS-2B cell lines were obtained from Istanbul University Faculty of Medicine, Physiology Department. Since they are commercially produced cell lines, no ethics committee approval is required.

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Competing Interests There is no competing interest.

Conflicts of interest Authors declare no conflicts of interest.

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